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A comparison of direct (flow cytometry) and indirect (stomatal guard cell lengths and chloroplast numbers) techniques as a measure of ploidy in black wattle, *Acacia mearnsii* (de Wild)

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Three assays, namely stomatal length measurements, counts of chloroplast numbers within the stomatal guard cells and flow cytometry, were used to confirm the ploidy of three diploid and three tetraploid *Acacia mearnsii* lines. The first two methods have previously been successful in identifying ploidy in this species, but they are indirect assessments. Flow cytometry directly quantifies the amount of DNA present in a sample, but has not been previously tested on *A. mearnsii*. It was decided to test the accuracy of all three methods by using the exact same plant material for each method. Results showed that each method correctly identified the ploidy of the diploid plant material tested as well as two of the three tetraploids tested. All three techniques identified the third tetraploid (C19/48/17) as a diploid, suggesting that its original classification was incorrect. From this study, all three techniques followed the same trend, confirming their accuracy for future research into ploidy identification of *A. mearnsii*. Flow cytometry has the advantage of providing a quick and efficient direct assessment of DNA in the sample, but is the most expensive. The other two techniques used (stomatal length measurements and counts of chloroplast numbers within the stomatal guard cells) are also accurate but are indirect methods and more time-consuming.

Introduction

Black wattle (*Acacia mearnsii* de Wild) was introduced into South Africa in 1864 (Beard 1957). Since then, it has become a leading commercially grown forestry tree species in South Africa. Originally grown for its tannin-rich bark for the leather-tanning industry, more recently black wattle has become an important source of high-quality raw material for pulp production. Therefore, the emphasis on black wattle breeding research in South Africa has shifted from producing high-quality bark with acceptable timber levels to improving timber quality with acceptable bark levels (Dunlop *et al.* 2003). Black wattle, however, has also been labelled as one of the top invader species of indigenous vegetation (Henderson 1989, 1992, 1998, Schumann and Little 1995, Turnbull *et al.* 1998, Joker 2000). Research aimed at restricting the spread of wattle outside of plantation boundaries and reducing the amount of seed contributing to the existing seedbank has therefore become a priority. One possible solution is to produce a polyploid individual. Due to the increased genome size and variety of possible chromosome pairings, the individual generally undergoes variable meiosis, with the result being unbalanced gametic chromosome numbers and hence sterility (Anon. 1974, Chaudhari and Barrow 1975, Ramsey and Schemske 1998).

When working with normal diploid and polyploid individuals, it has been observed that visible qualitative differences do occur between the two. Autotetraploids generally have larger nuclei and cell size compared to corresponding diploid individuals. This notable increase in cell volume of the autotetraploids is reflected in the dimensions of its phenotype or in its organs (Garber 1972). Einspahr *et al.* (1963) noted that triploid aspen (*Populus tremuloides*) had longer and wider fibres than their diploid counterparts. Bakulin (1980) showed that tetraploid aspen had longer cells than diploids, and similar such results were obtained by Van Buijtenen *et al.* (1959). More frequently-used methods of distinguishing autotetraploids from diploids involve stomatal guard cell length measurements, stomatal frequency per leaf surface area (Sax and Sax 1937, Evans 1955, Speckmann *et al.* 1965, Tan and Dunn 1973, Przywara *et al.* 1988, Beck *et al.* 2003a), counts of chloroplast numbers within the guard cells (Bingham 1968, Tan and Dunn 1973, Beck *et al.* 2003b) and pollen grain diameters (Najčevska and Speckmann 1968, Tan and Dunn 1973). However, the final decision as to the ploidy of the plant is dependent on the chromosome count (Garber 1972), as this is a direct assessment of the amount of DNA present in the individual. This becomes a problem with individuals having minute chromosomes, such as with various tree species (Bennett and Leitch 1995), black wattle included. Flow cytometry has, in more recent years, become a useful tool for the measurement of nuclear DNA content (Doležel *et al*. 1989, Sabharwal and Doležel 1993, Doležel *et al*. 1994, Harvey *et al*. 1995). It has proven to have a high degree of resolution and is a rapid and reliable technique (Doležel *et al*. 1989). It has been most commonly used for quantifying DNA in individuals where chromosome counts are unreliable due to their small size (Bennett and Leitch 1995). The advantage of flow cytometry is the ease and speed of sample preparation, allowing for large numbers of samples to be analysed. Flow cytometry also does not require cells that are dividing for DNA content analysis (Bennett and Leitch 1995). It is accurate in detecting small differences in genome size (Biradar and Rayburn 1994). This does not, however, suggest that in future there will be no need for cytological analysis. Rather, flow cytometry and cytological techniques should be used together (Bennett and Leitch 1995), as cytological studies are essential for robust interpretation of results.

In black wattle, especially the polyploids, the high number of chromosomes, combined with their small size and the low number of dividing cells that can be obtained from root tip squashes, leads to incorrect identification of polyploids. Various indirect techniques have been tested and have proven accurate and reliable in ploidy identification, namely stomatal length and frequency measurements (Beck *et al*. 2003a) as well as counts of chloroplast numbers within the stomatal guard cells (Beck *et al*. 2003b). However, these techniques, even though proven to be accurate and reliable, are still indirect assessments of ploidy, and therefore will always incorporate a degree of human error. It is therefore necessary to find an accurate, rapid and reliable technique which gives a direct measure of the amount of DNA present and thus allows future identification of black wattle polyploids.

This paper aims to compare two indirect techniques, already proven to be accurate assessments of ploidy levels, with a direct technique, namely flow cytometry.

Material and Methods

Plant material

Seeds from six different lines of *A. mearnsii* were collected and germinated under nursery conditions. The lines consisted of three diploids (117, 272, 283) and three colchicine-induced tetraploids (C19/48/17, C19/48/20, C4/49/52). The latter were obtained from experiments done earlier at the Wattle Research Institute (WRI) and confirmed via root tip squashes (WRI 1952). Fifteen seedlings from each line were selected and used in each of the three experiments. The pinnules of the first leaves were removed from one-month-old seedlings for the purposes of this investigation.

Stomatal guard cell length

A slide was prepared for each of the 15 seedlings within each of the six lines. Fifteen stomatal guard cell lengths were measured on each slide. For stomatal guard cell measurements, a razor blade was used to strip a thin layer from the abaxial surface of the pinnule. Initially, non-permanent mounts were prepared by floating the epidermal layers in a 1% aceto-carmine solution (w/v), covering with a cover slip. Examination with a light microscope followed, to check the quality of the epidermal sections. Permanent mounts were prepared by floating the epidermal layers in a 1% aceto-carmine solution and squashing with a cover slip. The cover slips were removed by soaking the slides in 45% acetic acid and, depending on whether the epidermal layers adhered to the slide or cover slip, they were passed through an alcohol dehydration series and mounted using Euparal essence and mountant. Stomatal guard cell lengths were measured under x40 magnification using a light microscope with an ocular scale, where each ocular unit of the scale measured 2.5µm.

Stomatal chloroplast counts

A razor blade was used to strip a thin layer from the abaxial surface of the pinnule, which was placed on a glass slide and mounted with a drop of stain (1:3 saturated solutions iodine:potassium iodide in 100ml of distilled water). Cover slips were placed on slides and viewed immediately, as samples degrade within 2h and visibility is retarded. A slide was prepared for each of the 15 seedlings within each of the six lines. Fifteen stomatal cells per plant were observed for chloroplast analysis using a light microscope at x40 magnification.

Flow cytometry analysis

Leaf samples from each of the 15 seedlings within each line were sent to the Agricultural Research Council (ARC), Institute for Tropical and Subtropical Crops, for flow cytometry analysis.

Statistical analysis

GenStat® Version 4.2 (Lane and Payne 1996) was used to statistically analyse the data. A general ANOVA was used to analyse the data. Chloroplast data were square-root transformed prior to analysis. The transformed means (μ) for these data were de-transformed (μ^2), making the values more practical. However, no least significant differences (LSD) or standard errors (SE) can be associated with these values (Rayner 1967). A correlation was done to compare all three methods tested.

Results

Stomatal guard cell length

Within each of the three diploid lines tested, there was little variation between plants. More variation was noted between plants within each of the tetraploid lines tested; however, this variation was not significant (Table 1). Mean stomatal guard cell length for the three diploid lines ranged from 24.3µm to 26.7µm, and for the tetraploid lines from 23.7µm to 34.9µm. One tetraploid line (C19/48/17) had significantly

Table 1: Guard cell lengths (μm) of *Acacia mearnsii* lines previously reported to be diploid and tetraploid (n = 15). Least significant difference between plants (1.98) and between lines (0.51) and standard error (1.01) were obtained from the ANOVA. Plant material unavailable for analysis is denoted by (*). Treatments denoted by the same letters are not significantly different

		Plant															
Ploidy	Line	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	Mean (μm)
	117	23.93	29.82	28.21	25.36	25.54	27.68	23.75	28.04	23.57	26.61	27.32	26.61	24.46	25.71	25.18	26.10 c
Diploid	272	26.43	26.79	27.86	26.79	26.25	26.25	28.21	26.79	26.25	29.29	25.54	25.00	25.89	25.89	27.32	26.70 d
	283	18.75	22.32	25.18	24.29	26.79	24.82	27.32	24.46	23.39	23.93	25.18	25.36	23.21	26.25	23.04	24.30 b
	C19/48/17	26.96	16.96	25.00	24.46	27.50	22.68	26.25	23.93	23.21	23.93	23.04	20.00	23.75	*	*	23.70 a
Tetraploid	C19/48/20	35.36	38.04	31.61	33.75	36.43	33.21	37.32	35.71	36.43	34.29	34.82	37.68	33.93	35.00	33.21	34.90 f
	C4/49/52	34.29	35.00	35.00	37.32	29.64	34.64	37.32	35.54	25.18	25.36	27.32	32.32	29.64	35.54	25.00	32.00 e

Table 2: Guard cell chloroplast counts of *Acacia mearnsii* lines previously reported to be diploid and tetraploid (n = 15). Data have been square-root transformed. Least significant difference between plants (0.22) and between lines (0.06) and standard error (0.11) were obtained from the ANOVA. De-transformed values (μ^2) are presented for the line means (μ), as these are more practical estimates; however, no LSD or SE can be associated with these values. Plant material unavailable for analysis is denoted by (*). Treatments denoted by the same letters are not significantly different

		Plant															Means	
Ploidy	Line	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	μ	μ^2
	117	2.65	2.79	2.69	2.63	2.49	2.50	3.15	2.39	.58	2.73	2.47	2.63	2.63	2.68	2.48	2.64 a	6.97 a
Diploid	272	2.79	2.47	2.58	2.17	2.43	2.13	2.63	2.31	2.55	2.40	2.72	2.44	2.99	2.63	2.57	2.52 b	6.35 b
	283	2.52	2.99	2.90	3.03	2.91	2.98	2.75	2.38	2.63	3.11	2.81	3.18	2.56	2.85	2.90	2.83 c	8.00 c
	C19/48/17	3.64	3.97	3.37	3.56	*	*	3.17	3.50	3.58	3.78	3.65	3.65	3.68	3.65	3.61	3.60 e	12.96 e
Tetraploid	C19/48/20	3.91	3.84	4.33	*	4.06	4.03	3.90	4.15	4.12	4.13	4.10	*	*	*	*	4.06 d	16.48 d
	C4/49/52	3.50	3.69	3.69	3.87	3.92	4.00	3.75	3.82	4.01	3.97	3.58	3.73	4.15	3.92	3.85	3.83 f	14.67 f

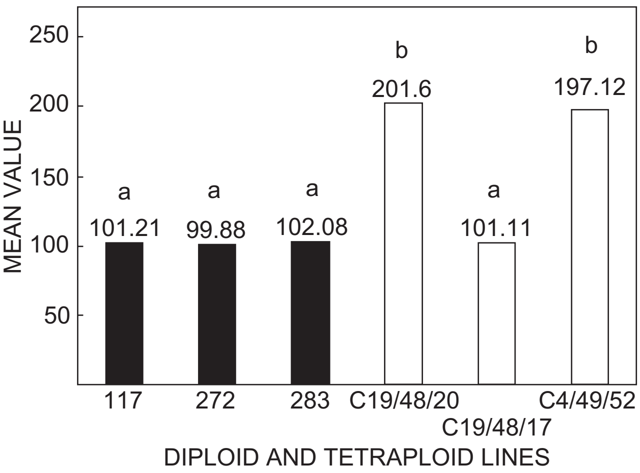

Figure 1: Differences in flow cytometry mean counts for six *Acacia mearnsii* lines previously reported to be diploid (■) or tetraploid (□). Treatments denoted by the same letters are not significantly different (LSD = 9.58)

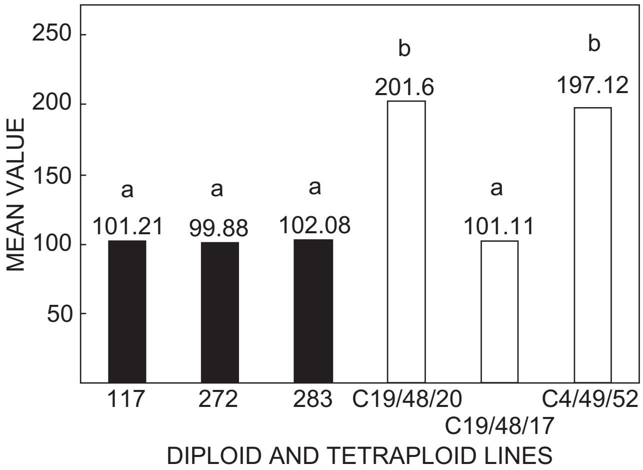

Figure 2: Comparison of stomatal guard cell length measurements, guard cell chloroplast numbers and flow cytometry analysis for *Acacia mearnsii* lines previously reported to be diploid (117, 272 and 283) or tetraploid (C19/48/20, C19/48/17 and C4/49/52)

shorter stomatal guard cell lengths, compared to those of the three diploid lines. Both other tetraploid lines had significantly longer stomatal guard cell lengths, compared to those of the three diploid lines tested ($P < 0.01$) (Table 1). Some plants within each of the tetraploid lines showed no significant differences, when their guard cell length was compared to that of some of the diploid plants.

Stomatal chloroplast counts

There was minimal variation noted within each of the six lines analysed (Table 2). The average number of chloroplasts in the guard cells of all the diploid lines was significantly less ($P < 0.01$) compared to that of all the tetraploid lines. The average number of chloroplasts per cell of the diploid lines ranged from 6.35 to 8.00, and for the tetraploids it ranged from 12.96 to 16.48 (Table 2).

Flow cytometry analysis

There was no variation noted between plants within each line tested. Significant differences were noted between the diploids and tetraploids ($P < 0.01$) (Figure 1), except for the one tetraploid line (C19/48/17) which showed no significant differences compared to that of the three diploid lines.

Discussion

When comparing all three methods used to analyse the same plant material, the same trend is apparent for each method used (Figure 2), and all three methods are highly correlated ($r > 0.604$), confirming the reliability of all three methods. In all three methods used, the tetraploid line C19/48/17 gave results that were unexpected. This line had stomatal lengths that were significantly shorter than the other tetraploid and diploid lines (Table 1), suggesting that it was not a tetraploid but rather a diploid. The counts of the chloroplast numbers (Table 2) showed that this line did have significantly ($P < 0.01$) more chloroplasts per stomatal guard cell than the three diploid lines, typical for a tetraploid. However, this count was significantly less than the other two tetraploid lines tested. Flow cytometry analysis classified this line to be a diploid (Figure 1). The fact that all three methods showed this line to be significantly different from the other tetraploid lines tested suggests that it is in fact a diploid line and was possibly misclassified in the early 1950s after tetraploid induction (Moffett and Nixon 1960).

The results from this paper are encouraging, as now an accurate, direct method (i.e. flow cytometry) has been shown to be able to identify the ploidy level of *A. mearnsii* samples. This method agrees with the two previously tested indirect methods, namely, stomatal length measurements and counts of chloroplast numbers within the stoma. The choice of method for ploidy identification for future work will depend on time and budget. Flow cytometry is quick and efficient but there is an expense involved for sample analysis; stomatal length measurements and counting chloroplast numbers within the stoma are inexpensive but time-consuming. However, all methods are accurate and have been shown to give the same results, regardless of genetic differences within and between lines.

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